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Expression of a polyubiquitin promoter isolated from *Gladiolus*

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Abstract A polyubiquitin promoter (*GUBQ1*) including its 5′UTR and intron was isolated from the floral monocot *Gladiolus* because high levels of expression could not be obtained using publicly available promoters isolated from either cereals or dicots. Sequencing of the promoter revealed highly conserved 5′ and 3′ intron splicing sites for the 1.234 kb intron. The coding sequence of the first two ubiquitin genes showed the highest homology (87 and 86%, respectively) to the ubiquitin genes of *Nicotiana tabacum* and *Oryza sativa RUBQ2*. Transient expression following gene gun bombardment showed that relative levels of GUS activity with the *GUBQ1* promoter were comparable to the CaMV 35S promoter in gladiolus, tobacco, rose, rice, and the floral monocot freesia. The highest levels of GUS expression with *GUBQ1* were attained with *Gladiolus*. The full-length *GUBQ1* promoter including 5′UTR and intron were necessary for maximum GUS expression in *Gladiolus*. The relative GUS activity for the promoter only was 9%, and the activity for the promoter with 5′UTR and 399 bp of the full-length 1.234 kb intron was 41%. *Arabidopsis* plants transformed with *uidA* under *GUBQ1* showed moderate GUS expression throughout young leaves and in the vasculature of older leaves. The highest levels of transient GUS expression in *Gladiolus* have been achieved using the *GUBQ1* promoter. This promoter should be useful for genetic engineering of disease resistance in *Gladiolus*, rose, and freesia, where high levels of gene expression are important.

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Introduction

Several promoters (*Arabidopsis UBQ3*, rice actin, *rolD*, mannopine synthase, translation elongation factor 1 subunit α, CaMV 35S, duplicated CaMV 35S, potato *Ubi3* and *Ubi7*, and phosphenolpyruvate carboxylase) have been examined for levels of stable expression in transgenic plants of *Gladiolus* (Kamo and Blowers 1999; Kamo et al. 2000). The highest level of GUS expression was from the CaMV 35S promoter even though *Gladiolus* is a monocot. The lowest levels of transient GUS expression in *Gladiolus* were with promoters such as rice *Act1* and maize *Ubi1* that were isolated from cereal monocots (Kamo et al. 1995). The maize *Ubi1* promoter has been used most frequently for developing transgenic plants of cereal monocots that show high constitutive levels of expression, but this promoter is not useful for high levels of expression in *Gladiolus*.

Control of gene expression appears to differ for the cereal and some of the floral and non-cereal monocots. In sugarcane, the maize *Ubi1* promoter showed higher levels of transient expression than the rice *Act1* and CaMV 35S promoters (Gallo-Meagher and Irvine 1993). In *Allium cepa*, onion, GUS activity was 5 × higher with the CaMV 35S promoter than with the rice *Act1* and maize *Ubi1* promoters following transient transformation (Eady et al. 1996). Wilmink et al. (1995) analyzed three members of the Liliaceae (Easter lily, leek, and tulips) and found that the cereal monocot promoters rice *Act1* and maize *Ubi1* performed well in rice, but not in leek cells. Rice *Act1* expressed well in tulip and lily leaves showing that there were differences in promoter strength among members of the same family. The maize *Ubi1* promoter performed as well as the CaMV 35S promoter in leek cells, better than the CaMV 35S in lily leaves, but poorly in tulip leaves. Addition of the maize *adh1* intron in the CaMV 35S promoter decreased expression in tulip, lily, and leek cells to

40–60% that of the CaMV 35S promoter without the intron. These conclusions on promoter strength were made following transient transformation rather than stable transformation. Our results with transient transformation have shown that low levels of GUS expression from promoters such as the rice *Act1* were confirmed in the transgenic *Gladiolus* plants. Promoters such as CaMV 35S, *rolD*, and *mas2* that showed high levels of transient GUS expression expressed at high levels in the transgenic *Gladiolus* plants, but the high levels of expression may be confined to specific tissues such as roots in the case of the *rolD* and *mas2* promoters.

It would be useful to have a promoter other than the CaMV 35S promoter that directs high levels of expression in *Gladiolus* and to understand elements of the promoter that affect gene expression in floral monocots. Only one promoter, the orchid cytokinin oxidase DSCKX1, has been isolated from a floral monocot, the orchid *Dendrobium* sp. (Yang et al. 2002). The polyubiquitin promoter has been isolated from several plants including *Arabidopsis* (Callis et al. 1990; Norris et al. 1993), sunflower (Binet et al. 1991), maize (Christensen et al. 1992), potato (Garbarino and Belknap 1994), *Nicotiana tabacum* (Genschik et al. 1994), rice (Wang et al. 2000; Wang and Oard 2003), parsley (Kawalleck et al. 1993), peas (Xia and Mahon 1998), and sugarcane (Wei et al. 2003; Yang et al. 2003). The levels of expression were higher for the polyubiquitin promoter than the CaMV 35S promoter in maize, rice, and *N. tabacum* so this promoter was chosen for isolation from *Gladiolus*. Expression of the polyubiquitin promoter (*GUBQ1*) isolated from *Gladiolus* was determined for freesia because it is in the same family as *Gladiolus* and for other floral monocots as well as representative woody, dicot, and cereal monocot species.

Materials and methods

Isolation of the ubiquitin promoter from *Gladiolus*

Genomic DNA was isolated from callus of *Gladiolus* cv. Jenny Lee using the method of Dellaporta et al. (1983) for constructing the genomic DNA library according to the procedure in the manual of the Universal Genome Walker Kit (www.clontech.com/clonetech). Genomic DNA was digested with *Dra* I, *Eco* RV, *Pvu* II, or *Stu* I and then ligated to the adaptors provided with the Genome Walker kit. Primers derived from a conserved sequence of the ubiquitin gene, UBQF-1: 5′-GGCCGYACCYTKGCKGACTAYAAYATC-3′ and UBQF-2: 5′-GCKGACTAYAAYATCCAGAAGGAGTC-3′, were used for PCR amplification of the Genome Walker library. This conserved sequence was determined by comparing the ubiquitin gene of several plants including *Pisum sativum*, *Zea mays*, and *Oryza sativa*. The first PCR reaction contained 1 μl (0.1 μg) of genomic DNA, 1 μl of a 10 μM solution of UBQF-1, 1 μl of a 10 μM solution of the AP1 adaptor primer (5′-GTAATACGACTCACTATAGGGC-3′), 5 μl of a 10 × buffer solution, 1 μl of a solution of dNTPs containing 10 mM of each nucleotide, 22 μl of a 25 mM solution of $Mg(OAc)_2$, 1 μl of the Advantage genomic polymerase mix (www.clontech.com/clontech), and 37 μl of distilled water. Amplification was done using seven cycles of 94°C for 25 s, 72°C for 3 min followed by 32 cycles of 94°C for 25 s, 67°C for 3 min followed by 67°C for 7 min with a Perkin and Elmer 480 DNA Thermal Cycler. The second round of PCR consisted of a PCR mixture of 1 μl of the first PCR reaction diluted 1:50, UBQF-2 and AP2 adaptor primers (5′-ACTATAGGGCACGCGTGGT-3′), and amplification was done using five cycles of 94°C for 25 s, 72°C for 3 min followed by 20 cycles of 94°C for 25 s, 67°C for 3 min followed by 67°C for 7 min. The PCR products were analyzed on a 1.5% agarose gel, and all bands resulting from PCR amplification were eluted using electroelution with dialysis tubing. Genomic DNA of *Gladiolus* that had been digested with either *Dra* I or *Eco* RV each resulted in two bands of interest that were 1.2 kb and 850 bp or 2.5 kb and 850 bp, respectively. Genomic DNA digested with either *Pvu* II or *Stu* I resulted in 400 and 500 bp bands, respectively. The sequence of the 1.2 kb band resulting from amplification of genomic DNA digested with *Dra* I was used to design primers for the next step of genome walking. Three steps of PCR amplification for genome walking were needed to obtain the sequence of the full-length promoter. All PCR products were ligated into a pGEM-T vector (www.promega.com) using AT cloning, transformed into *E. coli* DH5α, and then sequenced.

The full-length *GUBQ1* promoter was then isolated from genomic DNA of *Gladiolus* by PCR using the primers GubiP1-1 S (5′-ATCGGGCTAAACATAATGGGTGTTCTG-3′) and GubiP1-1 AS (CTGCGACAGAAAATGTATGGATCAGTA-3′). The PCR product was ligated into pGEM-T using AT cloning, transformed in *E. coli* DH5α, and then sequenced. The clone that contained the ubiquitin gene was named pG1-1.

The promoter sequence from *Gladiolus* in pG1-1 was subcloned into pUC-GUS using the *Sph* I/*Sal* I restriction sites from the pGEM-T vector to create a transcriptional fusion for transient GUS transformation assays (Fig. 1). pUC-GUS contains the *uidA*-nos terminator subcloned into the *Bam* HI/*Eco* RI sites of pUC 19. A series of promoter deletions (Figs. 1 and 2) were constructed by using PCR to isolate specific regions of the promoter from pG1-1 that were then subcloned into pUC-GUS. G1-2 was isolated using the primers GubiP1-1 S and GubiP1-2 AS (5′-TATCACAATTTAAACTTCACCGTTTCC-3′) and pG1-1 as the template DNA. The PCR product was subcloned into the pGEM-T vector. G1-2 was excised from the pGEM-T vector using *Sph* I/*Sal* I and then subcloned into pUC-GUS. G1-3 and G1-4 were subcloned using the same method as for G1-2 except that the primers used to isolate each deletion construct were different. G1-3 was isolated using the primers GubiP1-1 S and GubiP1-3 AS (5′-CTTTGAGAAATTTGGGGATAGAGAA-3′). G1-4 was isolated using the primers GubiP1-1 S and GubiP1-4 AS (5′-GAGAAAAGCACCGAAGACACCTAC-3′). All full-length and promoter DNA deletion constructs

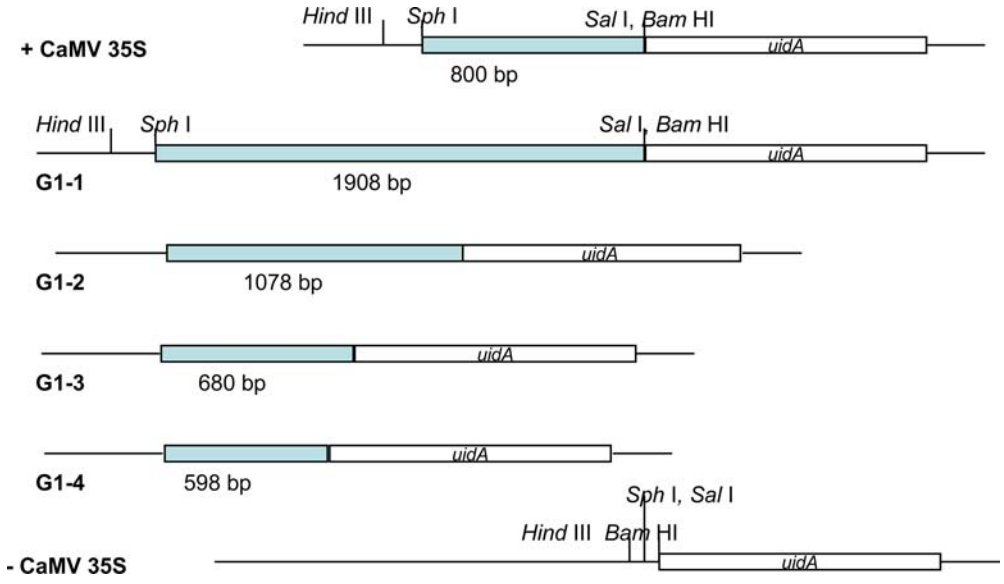

Fig. 1 Schematic of the DNA constructs used for transformation. *UidA* gene coding for GUS expression under control of various promoters shown from top to bottom: + 35S (CaMV 35S promoter), G1-1 (full-length ubiquitin promoter from *Gladiolus* consisting of the full-length 1.2 kb intron and 5′UTR of 80 bp), G1-2 (ubiquitin promoter, 5′UTR, and 399 bp of the intron), G1-3 (promoter and 5′UTR), G1-4 (promoter only lacking the 5′UTR and full-length intron), − 35S (*uidA* gene lacking a promoter)

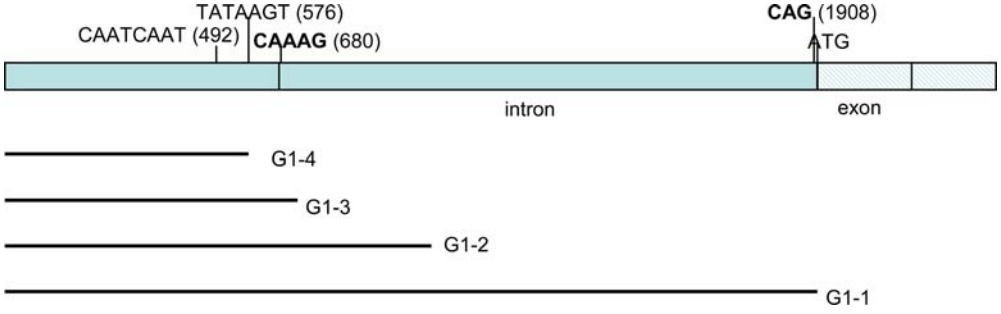

Fig. 2 Sequence of the ubiquitin promoter isolated from *Gladiolus* including two monomers of the ubiquitin coding sequence. The putative CAAT and TATA boxes are indicated. The 5′ and 3′ splice sites for the intron are in *bold type*. Numbers in parentheses are the nucleotide number. The region of the promoter included in each construct of the deletion series (G1-4, G1-3, G1-2, and G1-1) is shown

were sequenced to verify that PCR-generated mutations were not introduced and that the DNA inserts were in frame.

Plant cultures

Callus was initiated from in vitro-grown plants of *Zantedeschia eliotiana* cv. Flame (calla lily), that were grown on Murashige and Skoog's medium (MS; Murashige and Skoog 1962) supplemented with 3% sucrose, 0.2 mg/l Gelrite (www.sigmaaldrich.com), and the following in mg/l: glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; and 4-amino-3, 5, 6-trichloropicolinic acid (picloram, www.sigmaaldrich.com), 8.0. Calli of *Freesia* cv. Blue Lady were induced from floral stalks cultured on MS medium with 8 mg/l picloram. Bulb scales of *Lilium longiflorum* cv. Nellie White (Easter lily) were cultured on MS medium with 2 mg/l 3,6-dichloro-o-anisic acid (dicamba, www.sigmaaldrich.com) to induce callus. Floral stalks of *Canna* cv. Yellow King Humbert were cultured on MS with 2 mg/l 2,4-D to induce callus. Callus of calla lily, freesia, and cannas were maintained by monthly transfer to MS medium with 8 mg/l picloram. Calli of *O. sativa* cv. Taipei 309 (japonica rice) were maintained on N6 medium (Chu et al. 1975) supplemented with N6 vitamins, 3% sucrose, and the following in mg/l: casamino acids, 500; myo-inositol, 100; and 2,4-D, 2.0. Callus was induced from roots of in vitro-grown *Rosa hybrida* cv. Classy (hybrid tea rose) (Castillon and Kamo 2002). Rose calli were maintained on MS medium supplemented with 4 mg/l dicamba and 0.1 mg/l kinetin. In vitro-grown cormels of *Gladiolus* cv. Jenny Lee were sliced and cultured on MS medium with 2 mg/l 2,4-D to induce callus. Suspension cells were initiated from the callus of *Gladiolus* and maintained by transferring the suspension cells every 3 weeks to liquid MS medium with 2 mg/l 2,4-D at a 1:1 dilution. *N. tabacum* suspension cells were maintained in MS medium with 0.2 mg/l 2,4-D and 0.1 mg/l kinetin, and transferred weekly at a 1:37 dilution. The *Gladiolus* and *N. tabacum* suspension cells were grown on a gyratory shaker at 120 rpm. Callus and suspension cells were grown in the dark at 25°C.

Arabidopsis transformation

The *GUBQ1* promoter was excised from pG1-1 using *Hind* III and *Bam* HI and then ligated into pCAMBIA 1391Z that had been cut with *Hind* III and *Bam* HI. The pCAMBIA 1319Z vector (received from the Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia) is designed for promoter testing in plants as it contains a *uidA* gene with the catalase intron and *hptII* for hygromycin selection of plants. This plasmid was then used to transform *Agrobacterium tumefaciens* C5851. Transformation of *Arabidopsis thaliana* ecotype Columbia plants that had been grown 5 weeks in a growth chamber with a 16 h photoperiod at 25°C was accomplished by vacuum infiltration (Bechtold and Pelletier 1998). Hygromycin-resistant T1 plants were selected by planting seeds on MS medium supplemented with 30 mg/l hygromycin. The selected seedlings were transferred to soil.

Plasmid DNAs and bombardment

Plasmid DNA was purified using alkaline lysis followed by cesium chloride gradient purification (Maniatis et al. 1982) and used for bombardment.

The PDS-1000 He system (www.bio-rad.com) was used for delivery of 1.0 μm gold particles to suspension cells. Gold particles were coated with plasmid DNA according to Sanford et al. (1993). Each DNA construct to be tested was co-bombarded with pD0432 containing the *luc* gene coding for luciferase expression under control of the CaMV 35S promoter (Ow et al. 1986). Three hours prior to bombardment gladiolus, freesia, rose, and tobacco cells were placed in liquid MS medium containing osmoticum in addition to the same hormone concentration as used for callus or suspension cell maintenance. Rose and tobacco cells were placed in liquid MS medium containing 0.25 M mannitol. Freesia and gladiolus cells were placed in liquid MS medium containing 0.125 M mannitol. After 3 h of incubation in liquid MS medium with osmoticum, cells were plated on Whatman no. 1 filter paper over solid MS medium containing the same osmoticum concentration.

Rose and lily cells were bombarded at 900 psi. Freesia, tobacco, rice, calla lily, and cannas were bombarded at 1100 psi, and gladiolus cells at 1200 psi (8.3 mPA). Each plate was bombarded once at a target distance of 12 cm and then incubated at 25°C in the dark.

UidA expression

Specific activity of *uidA* expression was measured by fluorometric determination of methylumbelliferone (4-MU) according to Jefferson et al. (1987). Two days following bombardment, all cells on the bombarded Petri plate were ground in extraction buffer (100 mM KH_2PO_4, pH 7.0, and 1 mM dithiothreitol). All cells, except the Easter lily and calla lily cells, were placed in FASTDNA tubes (www.qbiogene.com) containing a ceramic ball and extraction buffer for homogenization using the FastPrep Instrument (www.qbiogene.com). The Easter lily and calla lily cells were ground on ice using a mortar and pestle. Following centrifugation at 16,000 × *g* at 4°C for 12 min, an aliquot of the supernatant was used to determine luciferase activity on the day of extraction. Another aliquot was stored at −70°C, until it was used for the fluorometric determination of GUS activity by adding the cell extract to the assay buffer (1 mM methylumbelliferyl-β-D-glucuronide) for incubation at 37°C. Aliquots of the assay buffer including sample were added to 0.2 M sodium carbonate after 0, 15, 30, and 60 min of incubation. Fluorescence was measured with a BioRad VersaFluor Fluorometer set at 360/40 nm for excitation and 460/10 nm for emission. Protein concentration in the tissue extract was measured using the bicinchoninic (BCA) protein assay reagent (www.piercenet.com) according to the manufacturer's instructions.

Three Petri plates of cells for each replicate, and three replicates were bombarded for determining GUS expression of each DNA construct tested. GUS activity was reported as relative GUS activity and calculated by dividing the GUS activity measured in nmol/min/mg protein by the transformation efficiency factor derived from the measured luciferase activity (Schledzewski and Mendel 1994). An analysis of variance ($P<0.001$) followed by Dunn's multiple comparison with a 95% confidence interval ($P \leq 0.05$) was performed using Sigmastat (www.systat.com) to compare the means of the relative GUS activity for each DNA construct.

Luciferase expression

An aliquot of the supernatant (30 μl) following grinding and centrifugation of the cells in extraction buffer and 5 μl of 4 mM sodium pyrophosphate were added to 100 μl of assay buffer (30 mM Tricine, 3 mM ATP, 15 mM $MgSO_4$, 10 mM dithiothreitol), followed by addition of 100 μl of D-luciferin (4 mM), and the tube was immediately read in a 20/20 Luminometer (www.turnerbiosystems.com).

Results and discussion

Structure of the *GUBQ1* promoter

A Genome Walker library of *Gladiolus* that was screened with primers to a conserved region of the ubiquitin gene resulted in the isolation of the *GUBQ1* promoter and two monomers of the polyubiquitin gene (GenBank accession number DQ445914). Sequencing of the promoter revealed a 1.234 kb intron with highly conserved 5′ and 3′ splicing sites (Fig. 2). The 5′ splicing site of *GUBQ1* is AAAG gta showing one nucleotide mismatch from the conserved sequence CAAG gta. The sequence of the 3′ splicing site, CAG, is identical to that found in other plants (Kawalleck et al. 1993; Genschik et al. 1994; Wang et al. 2000). The putative CAAT and TATA boxes are indicated in Fig. 2.

Table 1 Transient GUS expression for the full-length and deletions of the *GUBQ1* promoter in *Gladiolus* suspension cells

Promoter	Relative GUS activity[a] (nmol/min/mg·rlu)	Relative expression[b]
− 35S	11.31 ± 3.42 a	0.08
Maize *Ubi1*	12.36 ± 0.86 a	0.09
+ 35S	132.34 ± 52.38 b	1.00
G1-1	420.33 ± 167.94 b	3.18
G1-2	173.84 ± 71.33 b	1.31
G1-3	99.60 ± 49.90 c	0.75
G1-4	37.20 ± 10.32 c	0.28

[a]Values with different letters are significantly different at $P \leq 0.05$ according to Dunn's Method. Standard errors are shown here

[b]Values represent relative GUS expression levels where 35S-GUS is set at 1.00

The location of the 5′ intron is highly conserved, and it is immediately upstream of the ATG (Norris et al. 1993).

Introns derived from cereal monocots have a lower AU content than that of dicots (Goodall and Filipowicz 1991; Norris et al. 1993). The intron of *GUBQ1* has an AU content of 68%, which is characteristic of a dicot intron that contains a minimum 60% AU and an average of 74% AU. Introns from cereal monocots contained an average of 59% AU (Goodall and Filipowicz 1991). The 457 bp intron of the *N. tabacum* polyubiquitin gene contains an AU content of 72% (Genschik et al. 1994). Four polyubiquitin genes have been isolated from peas and the *PUB1* intron of 584 bp has a 71.4% AU content. *PUB2* has an intron of 687 bp with a 71.9% AU content. *PUB3* is 787 bp with 70.4% AU. *PUB4* is 1114 bp with a 65.8% AU content. The *ubi*4-2 gene of *Petroselinum crispum*, parsley, contains a 587 bp intron (Kawalleck et al. 1993). *Ubi7* from potato has an intron of 569 bp (Garbarino et al. 1995). The introns from *RUBQ1* and *RUBQ2* of rice were 782 and 962 bp, respectively (Wang et al. 2000). The *Ubi1* intron in the 5′ UTR region of maize is 1010 bp and the intron of *Ubi2* is 3.1 kb (Christensen et al. 1992).

The polyubiquitin genes contain tandem repeats of the 228 bp coding region that have high homology to each other (Callis et al. 1990). The coding sequences for rice *RUBQ1* and *RUBQ2* showed 88% homology with the maize polyubiquitin gene, 81% to the *A. thaliana*, and 82% to the sunflower ubiquitin genes. The coding sequence of two ubiquitin monomers of *GUBQ1* showed 87% homology to *N. tabacum* (GenBank accession no. AF154647.1) and 86% homology to the *O. sativa* polyubiquitin gene *RUBQ2* (GenBank accession no. AF184280).

Deletion analysis of *GUBQ1*

The full-length *GUBQ1* promoter showed GUS expression levels that were 30 × higher than the maize *Ubi1* promoter and comparable to the CaMV 35S promoter in suspension cells of *Gladiolus* bombarded for transient transformation (Table 1, Fig. 3). The relative GUS activity was statistically

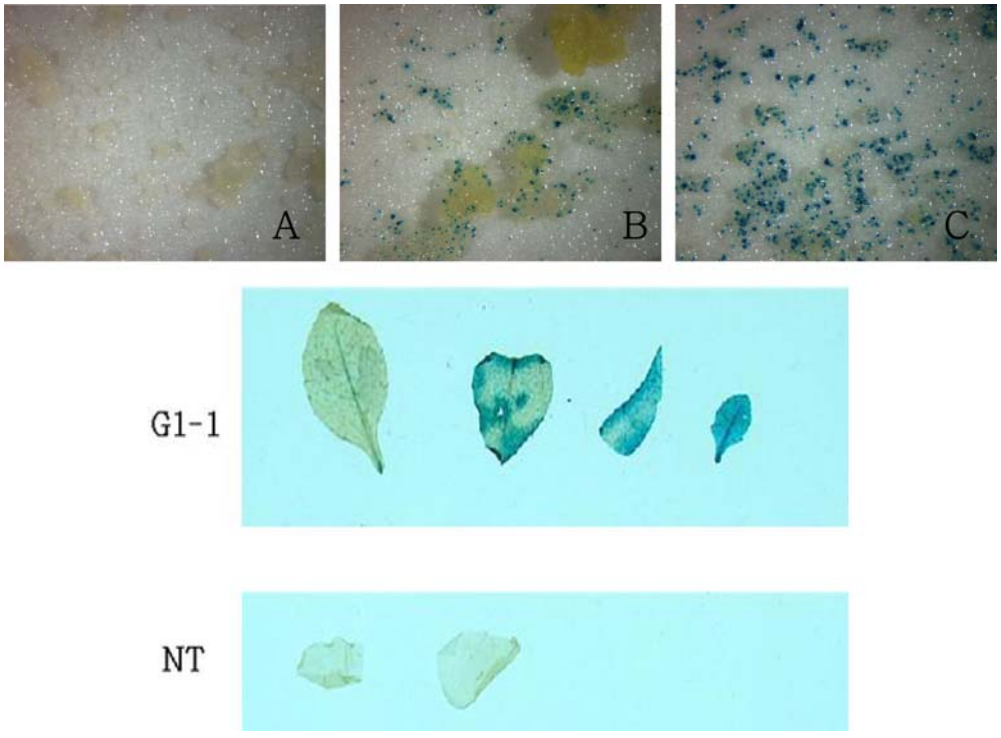

Fig. 3 Suspension cells of *Gladiolus* showing GUS expression 48 h following bombardment with (**A**) − 35S, (**B**) + 35S, or (**C**) G1-1 plasmid DNAs. The relative GUS activity was statistically comparable for the CaMV 35S and full-length *GUBQ1* promoters even though the relative GUS activity was consistently 2.5–3.0 × higher for the *GUBQ1* promoter (**C**) as compared to the CaMV 35S promoter (**B**), as seen following histochemical staining. Leaves from *Arabidopsis* transformed with the *uidA* gene under the *GUBQ1* promoter (*top*). Non-transformed plant (*bottom*)

Table 2 Comparison of the *GUBQ1* promoter activity in various floral monocots

Plant	Promoter	Relative GUS activity[a] (nmol/min/mg·rlu)	Relative GUS expression[b]
Calla lily	− 35S	38.56 ± 11.85 a	0.54
	+ 35S	71.47 ± 30.20 a	1.00
	G1-1	36.11 ± 13.51 a	0.50
	G1-4	12.89 ± 3.52 a	0.18
Freesia	− 35S	10.61 ± 1.64 a	0.44
	+ 35S	24.21 ± 4.68 b	1.00
	G1-1	21.56 ± 2.28 b	0.89
	G1-4	14.94 ± 2.87 b	0.62
Cannas	− 35S	41.76 ± 7.81 a	0.10
	+ 35S	435.26 ± 185.11 b	1.00
	G1-1	79.40 ± 16.07 c	0.18
	G1-4	73.30 ± 33.66 c	0.17
Easter lily	− 35S	43.54 ± 10.22 a	0.64
	+ 35S	68.40 ± 10.60 a	1.00
	G1-1	55.10 ± 15.65 a	0.81
	G1-4	52.17 ± 12.74 a	0.76

[a]Values with different letters are significantly different at $P \leq 0.05$ according to Dunn's Method. Standard errors are shown here

[b]Values represent relative GUS expression levels where 35S-GUS is set at 1.00

Table 3 Comparison of the *GUBQ1* promoter activity in a cereal monocot (rice), a dicot (tobacco), and a woody plant (rose)

Plant	Promoter	Relative GUS activity[a] (nmol/min/mg·rlu)	Relative GUS expression[b]
Rice	− 35S	25.00 ± 5.19 a	0.08
	+ 35S	299.50 ± 103.94 b	1.00
	G1-1	63.97 ± 19.58 b	0.21
	G1-4	55.16 ± 20.48 b	0.18
Tobacco	− 35S	19.64 ± 3.06 a	0.23
	+ 35S	86.64 ± 31.40 b	1.00
	G1-1	115.89 ± 42.12 b	1.34
	G1-4	135.98 ± 53.99 b	1.57
Rose	− 35S	9.81 ± 2.02 a	0.36
	+ 35S	26.98 ± 4.60 b	1.00
	G1-1	60.12 ± 16.05 b	2.23
	G1-4	14.25 ± 5.02 c	0.53

[a]Values with different letters are significantly different at $P \leq 0.05$ according to Dunn's Method. Standard errors are shown here

[b]Values represent relative GUS expression levels where 35S-GUS is set at 1.00

comparable for the CaMV 35S and full-length *GUBQ1* promoter. The relative GUS activity was 2.5–3.0 × higher for the *GUBQ1* promoter as compared to the CaMV 35S promoter in bombarded cells from all three replicates, and this relatively higher GUS activity for the *GUBQ1* promoter was visually obvious following histochemical staining (Fig. 3). Differences in GUS expression observed following histochemical staining were often more revealing than the measurements of relative GUS activity because of the limit of detection when measuring the specific activity of *uidA* expression. For example, there were very small blue spots on calla lily and Easter lily cells following bombardment with the *GUBQ1* promoter, and there were very light blue spots on *Gladiolus* cells following bombardment with the maize *Ubi1* promoter. The relative GUS activity of these three samples was not significantly different from the cells bombarded with the vector lacking a promoter although histochemical staining revealed the presence of cells expressing GUS. Unfortunately, there were too many cells expressing GUS following staining to making counting them feasible.

Elimination of both the intron and 5′UTR by using the G1-4 DNA construct for gene gun bombardment resulted in only 9% of the GUS expression level of the full-length promoter that included the 5′UTR and intron. This difference in GUS expression was statistically significant (Table 1). Addition of the 5′UTR to the promoter by using the G1-3 DNA construct for bombardment resulted in 24% of the full-length promoter's level of GUS expression. This was a statistically significant difference in GUS expression when compared to the level of expression when cells were bombarded with either G1-2 that consists of the promoter, 5′UTR, and 30% of the 5′ region of the intron or G1-1 that is the full-length promoter. These results indicated that the intron contributed to GUS expression but not to the extent reported by others for the cereal monocots. Wang and Oard (2003) reported that deletion of the *RUBQ2* intron reduced expression to background levels. In cereal monocots, the intron has a tremendous effect on affecting levels of gene expression as shown for the rice actin and other promoters (McElroy et al. 1990). Our previous results showed that the *Arabidopsis* intron $UBQ3_{I1}$ added to the CaMV 35S promoter stimulated transient GUS activity in *Gladiolus* suspension cells by 6 × (data not shown). In *N. tabacum* deletion of the intron did not affect transient expression in protoplasts (Genschik et al. 1994). Deletion of the intron from *Arabidopsis UBQ3*, *UBQ10*, and *UBQ11* resulted in 2.5–3 × lower transient luciferase activity in *Arabidopsis* leaves (Norris et al. 1993).

Expression of *GUBQ1* in other plant species

Callus and suspension cells of several other floral monocots were used for transient transformation with either G1-1 or G1-4 to determine if levels of expression were comparable to that in *Gladiolus* and if expression was affected by the intron and 5′UTR. Freesia is in the same family, the Iridaceae, as *Gladiolus*. GUS expression in freesia of the full-length *GUBQ1* was similar to that of the CaMV 35S promoter, and the relative GUS activity in freesia was only 5% of that in *Gladiolus* (Tables 1 and 2). The relative GUS activity was 5 × higher for the CaMV 35S promoter as compared to *GUBQ1* in cannas. Deletion of the intron and 5′UTR from *GUBQ1* did not affect the relative GUS expression of *GUBQ1* in freesia, calla lily and cannas. Both calla lily and cannas are evolutionarily distant from *Gladiolus* (Chase 2004). In Easter lily, the relative GUS expression of *GUBQ1* was similar to that of the CaMV 35S promoter.

Transgenic *Arabidopsis* plants containing *uidA* under control of the *GUBQ1* promoter showed moderate GUS expression throughout leaves, particularly in the veins. The blue staining indicative of GUS expression was strongest around the wounded areas on the leaf indicating a possible wound-inducible expression (Fig. 3).

This is the second study on gene expression from a promoter derived from an ornamental, non-cereal monocot, and expression was examined in cells of a representative cereal monocot (rice), dicot (*N. tabacum*), and woody plant (rose) (Table 3). *GUBQ1* expressed at levels comparable to the CaMV 35S promoter in tobacco, and there was no effect of the intron on expression. *GUBQ1* expressed as well as the CaMV 35S promoter in rice, and as the CaMV 35S promoter is known to express poorly as compared to the *Act1* promoter in rice, this indicates that *GUBQ1* is not a useful promoter for rice. The levels of GUS expression with the *GUBQ1* promoter were comparable to the CaMV 35S promoter in rose cells although the levels of GUS were 1.7–2.0 × higher in cells bombarded in all three replicates (Table 3). The comparable expression of the CaMV 35S and *GUBQ1* promoters in other systems suggests that *GUBQ1* may be useful for gene expression in rose and other cells that express well with the CaMV 35S promoter.

Wilmink et al. (1995) showed that members of the Liliaceae had promoter preferences that were more similar to tobacco than the cereal monocots, and this was observed in our studies with *Gladiolus* and other plant species using a promoter isolated from *Gladiolus*. It was not surprising that the sequence of *GUBQ1* revealed an intron with a high AU content similar to that of a dicot. *GUBQ1* will be useful for genetic engineering of *Gladiolus* and possibly rose and freesia when high levels of gene expression are critical.

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References

Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol Biol 82:259–266

Binet MN, Weil JH, Tessier LH (1991) Structure and expression of sunflower ubiquitin genes. Plant Mol Biol 17:395–407

Callis J, Raasch JA, Vierstra RD (1990) Ubiquitin extension proteins of *Arabidopsis thaliana:* structure, localization, and expression of their promoters in transgenic tobacco. J Biol Chem 21:12486–12493

Castillon J, Kamo K (2002) Maturation and conversion of somatic embryos of three genetically diverse rose cultivars. Hort Sci 37:973–977

Chase M (2004) Monocot relationships: an overview. Am J Bot 91:1645–1655

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Eady CC, Lister CE, Suo Y, Schaper D (1996) Transient expression of *uidA* constructs in in vitro onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery. Plant Cell Rep 15:958–962

Gallo-Meagher M, Irvine JE (1993) Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Rep 12:666–670

Garbarino JE, Oosumi T, Belknap WR (1995) Isolation of a polyubiquitin promoter and its expression in transgenic potato plants. Plant Physiol 109:1371–1378

Garbarino JE, Belknap WR (1994) Use of ubiquitin promoters for transgene expression in potato. In: Belknap WR, Vayda ME, Park WD (eds) Molecular and cellular biology of the potato, 2nd edn. CAB International, Wallingford, UK, pp 173–185

Genschik P, Marbach J, Uze M, Feuerman M, Plesse B, Fleck J (1994) Structure and promoter activity of a stress and developmentally regulated polyubiquitin-encoding gene of *Nicotiana tabacum*. Gene 148:195–203

Goodall GJ, Filipowicz W (1991) Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. EMBO J 10:2635–2644

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kamo K, Blowers A, Smith F, Van Eck J, Lawson R (1995) Stable transformation of *Gladiolus* using suspension cells and callus. J Am Hortic Sci 120:347–352

Kamo K, Blowers A (1999) Tissue specificity and expression level of *gusA* under *rolD*, mannopine synthase and translation elongation factor 1 subunit α promoters in transgenic *Gladiolus* plants. Plant Cell Rep 18:809–815

Kamo K, Blowers A, McElroy D (2000) Effect of the cauliflower mosaic virus 35S, actin, and ubiquitin promoters on *uidA* expression from a *bar-uidA* fusion gene in transgenic *Gladiolus* plants. In Vitro Cell Dev Biol Plant 36:13–20

Kawalleck P, Somssich IE, Feldbrugge M, Hahlbrock K, Weisshaar B (1993) Polyubiquitin gene expression and structural properties of the *ubi*4-2 gene in *Petroselinum crispum*. Plant Mol Biol 21:673–684

Maniatis T, Fritsch F, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163–171

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Norris SR, Meyer SE, Callis J (1993) The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol Biol 21:895–906

Ow D, Wood KV, DeLuk M, Wet JR, Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plants cells and transgenic plants. Science 234:856–859

Sanford JC, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol 217:483–510

Schledzewski K, Mendel RR (1994) Quantitative transient gene expression: comparison of the promoters for maize polyubiquitin1, rice actin1, maize-derived *Emu* and CaMV 35S in cells of barley, maize and tobacco. Trans Res 3:249–255

Wang J, Jiang J, Oard JH (2000) Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). Plant Sci 156:201–211

Wang J, Oard JH (2003) Rice ubiquitin promoters: deletion analysis and potential usefulness in plant transformation systems. Plant Cell Rep 22:129–134

Wei H, Wang ML, Moore PH, Albert HH (2003) Comparative analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants. J Plant Physiol 160:1241–1251

Wilmink A, van de Ven BCE, Dons JJM (1995) Activity of constitutive promoters in various species from the Liliaceae. Plant Mol Biol 28:949–955

Xia X, Mahon J (1998) Pea polyubiquitin genes: (I) structure and genomic organization. Gene 215:445–452

Yang M, Bower R, Burow MD, Paterson AH, Mirkov TE (2003) Genomics, molecular genetics and biotechnology. Crop Sci 43:1805–1813

Yang SH, Yu H, Goh CH (2002) Isolation and characterization of the orchid cytokinin oxidase DSCKX1 promoter. J Exp Bot 53:1899–1907